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Characterization and functional expression in *Escherichia coli* of the sodium/proton/glutamate symport proteins of *Bacillus stearothermophilus* and *Bacillus caldotenax*

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Summary

The genes encoding the Na⁺/H⁺/L-glutamate symport proteins of the thermophilic organisms *Bacillus stearothermophilus* (*gltT*$_{Bs}$) and *Bacillus caldotenax* (*gltT*$_{Bc}$) were cloned by complementation of *Escherichia coli* JC5412 for growth on glutamate as sole source of carbon, energy and nitrogen. The nucleotide sequences of the *gltT*$_{Bs}$ and *gltT*$_{Bc}$ genes were determined. In both cases the translated sequences corresponded with proteins of 421 amino acid residues (96.7% amino acid identity between GltT$_{Bs}$ and GltT$_{Bc}$). Putative promoter, terminator and ribosome-binding-site sequences were found in the flanking regions. These expression signals were functional in *E. coli*. The hydropathy profiles indicate that the proteins are hydrophobic and could form 12 membrane-spanning regions. The Na⁺/H⁺ coupled L-glutamate symport proteins GltT$_{Bs}$ and GltT$_{Bc}$ are homologous to the strictly H⁺ coupled L-glutamate transport protein of *E. coli* K-12 (overall 57.2% identity). Functional expression of glutamate transport activity was demonstrated by uptake of glutamate in whole cells and membrane vesicles. In accordance with previous observations (de Vrij *et al.*, 1989; Heyne *et al.*, 1991), glutamate uptake was driven by the electrochemical gradients of sodium ions and protons.

Introduction

To date, two types of L-glutamate transport mechanisms have been reported for thermophilic bacteria. In *Bacillus stearothermophilus* L-glutamate (or L-aspartate) transport proceeds via a sodium/proton-symport mechanism with a 1:1:1 stoichiometry (de Vrij *et al.*, 1989; 1990; Heyne *et al.*, 1991). In *Clostridium fervidus* an electrogenic sodium

symport mechanism with a stoichiometry of 2 has been identified (Speelmans *et al.*, 1989). In the mesophilic organism *Escherichia coli* three L-glutamate transport systems have been identified: (i) a binding-protein-dependent, sodium-independent, glutamate–aspartate system (inhibited by cysteate); (ii) a binding-protein-independent, sodium-independent, glutamate–aspartate system (inhibited by β-hydroxyaspartate and cysteate; and (iii) a binding-protein-independent, sodium-dependent, glutamate-specific system (inhibited by α-methylglutamate) (Halpern *et al.*, 1973; Miner and Frank, 1974; Schellenberg and Furlong, 1977). Genes encoding the sodium-motive and proton-motive transport systems, designated *gltS* and *gltP*, respectively, have been cloned (Deguchi *et al.*, 1989; Kalman *et al.*, 1991; Wallace *et al.*, 1990) and their nucleotide sequences have been reported (Deguchi *et al.*, 1990; Kalman *et al.*, 1991; Wallace *et al.*, 1990). Recently the reported sequence of the *gltP* gene of *E. coli* has been corrected (Tolner *et al.*, 1992).

The mechanism of energy coupling to glutamate transport in *B. stearothermophilus* has been described (de Vrij *et al.*, 1989; Heyne *et al.*, 1991). To elucidate the molecular properties of the sodium/proton/L-glutamate-symport transport system of *B. stearothermophilus* in more detail, a strategy was devised to clone the gene encoding the glutamate transport protein. This strategy is based on the complementation of an *E. coli* K-12 strain for growth on glutamate as sole source of energy, nitrogen and carbon. *E. coli* K-12 strains do not grow in media containing glutamate as sole source of energy, nitrogen and carbon because of an insufficient capacity to accumulate glutamate (Halpern and Lupo, 1965). Another thermophilic bacillus is *Bacillus caldotenax*, which has a higher optimum temperature of growth (70 versus 63°C of *B. stearothermophilus*), and can grow much faster than *B. stearothermophilus* on glutamate as sole source of energy, nitrogen and carbon (t$_d$: 30 min versus 5 h for *B. stearothermophilus*). The L-glutamate transport gene of *B. caldotenax* has also been isolated and characterized.

In this paper we report the cloning of the genes encoding the Na⁺/H⁺/L-glutamate symport proteins of *B. stearothermophilus* (*gltT*$_{Bs}$) and *B. caldotenax* (*gltT*$_{Bc}$), their nucleotide sequence, deduced amino acid sequence
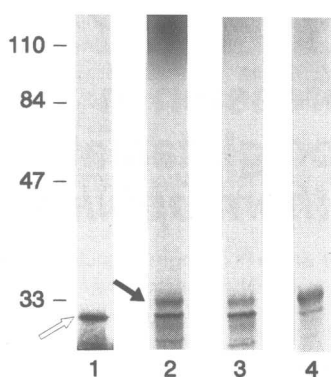
Fig. 1. Expression of the *gltT* genes of *B. stearothermophilus* and *B. caldotenax* in minicells of *E. coli* P678–54. Proteins were labelled in the presence of [^{35}S]-methionine (>1000 Ci mmol^{-1}) and 100 µM IPTG, and separated on a 15% SDS/PAA gel. Lanes 1–4: P678–54 containing pUC18 (vector control), pGBT231 (GltT$_{Bc}$), pGBT102 (GltT$_{Bs}$), and pGBT112 (GltT$_{Bs}$ expressed in the opposite direction from the *lac* promoter), respectively. Molecular size markers (in kDa) are indicated. Solid arrow: glutamate transport proteins. Open arrow: product of the ampicillin-resistance gene.

and deduced hydropathy profile. We conclude that GltT$_{Bs}$ and GltT$_{Bc}$ are homologous. Furthermore, these proteins are homologous to the H$^+$/glutamate symport protein of *E. coli* K-12.

Results

Cloning of the glutamate transport genes of *B. stearothermophilus* and *B. caldotenax*

The *gltT*$_{Bs}$ and *gltT*$_{Bc}$ genes were cloned using the strategy outlined in the *Experimental procedures*. In the case of *gltT*$_{Bs}$ 61 Glu$^+$ transformants able to grow on M9G plates (supplemented with carbenicillin and IPTG) were collected after 48 h. The cells were grown in liquid media

and their plasmid content was analysed with respect to insert size. All transformants did harbour plasmid pKK223–3, with inserts ranging from 2.5 to 7 kb in length. A total of 30 of these plasmids conferred a Glu$^+$ phenotype on *E. coli* JC5412 upon retransformation. One transformant harbouring pGBT38 (insert 2.5 kb) was used to perform uptake experiments in whole cells. In these cells, sodium-stimulated glutamate transport activity was significantly higher than in cells harbouring plasmid pKK223–3 (data not shown). To obtain the smallest insert that allowed JC5412 to grow on M9G, subclones of pGBT38 were constructed in pUC18. The two smallest hybrid plasmids which resulted in a Glu$^+$ phenotype of JC5412, were pGBT102 and pGBT112. Both plasmids contained a 1537 bp *Eco*RI fragment of pGBT38 but in opposite orientation. Since the *gltT*$_{Bs}$ gene in pGBT112 is expressed in the opposite direction of the *lac* promoter, the *gltT*$_{Bs}$ promoter may have been cloned along with the *gltT*$_{Bs}$ gene.

The *gltT*$_{Bc}$ gene was cloned essentially as described above for the *gltT*$_{Bs}$ gene, and was located on a 1535 bp *Eco*RI fragment (pGBT231). The *gltT*$_{Bc}$ gene could also be expressed independently of its orientation relative to the *lac* promoter of pUC18.

Expression of the glutamate transport genes of *B. stearothermophilus* and *B. caldotenax*

In the minicell-producing strain *E. coli* P678–54, in which pGBT102 and pGBT112 were used to express GltT$_{Bs}$, one additional protein band with an apparent molecular mass of 33 kDa was found which was not present in a control strain containing pUC18 (Fig. 1).

Uptake of L-glutamate and L-aspartate by whole cells (strain *E. coli* JC5412) harbouring pGBT102 (GltT$_{Bs}$) was several-fold higher than in cells harbouring pUC18 (Fig. 2). The initial rate of uptake and steady-state level of
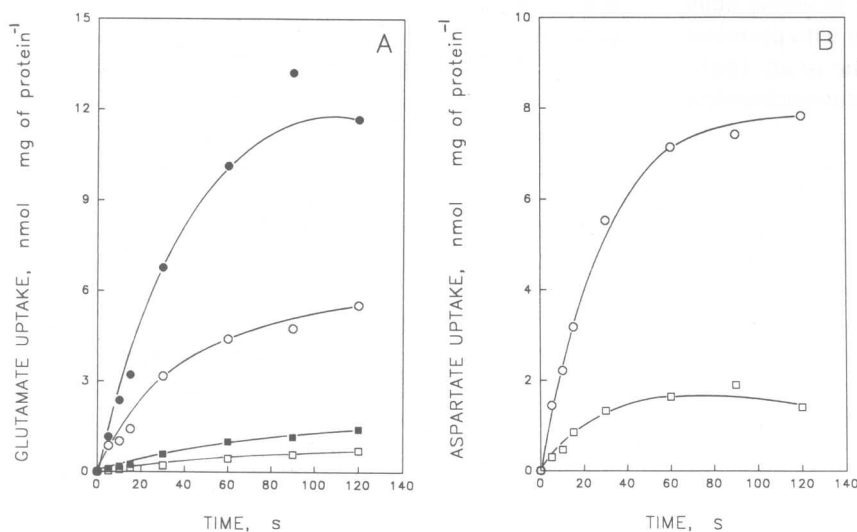
Fig. 2. Sodium-ion dependent uptake of L-glutamate (A) and L-aspartate (B) by cells expressing the glutamate transport protein of *B. stearothermophilus*. Uptake by cells harbouring plasmid pUC18 (vector control; □, ■,) and pGBT102 (GltT$_{Bs}$; O, ●,) were compared. Concentrated cells were diluted to a final concentration of 0.75 mg protein per ml into 50 mM potassium phosphate, pH 6.0, 5 mM MgSO$_4$, and 10 mM glucose buffer, with (●, ■,) or without (O, □,) 20 mM NaCl. After 1 min of incubation, L-[^{14}C]-glutamate (1.75 µM) or L-[^{14}C]-aspartate (2.23 µM) was added and transport assays were further handled as described in the *Experimental procedures*.
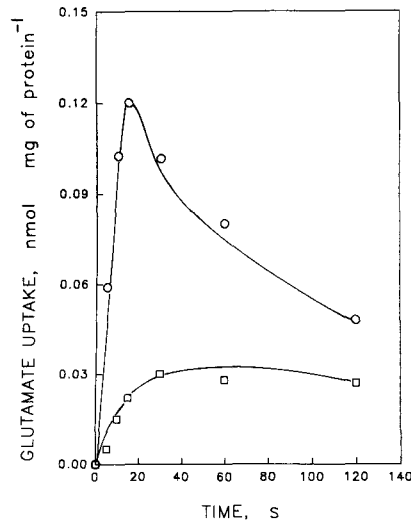
Fig. 3. L-glutamate counterflow activity by membrane vesicles of *E. coli* (JC5412(pUC18 or pGBT102)). Counterflow by membrane vesicles prepared from *E. coli* harbouring either pUC18 (vector control; □) or pGBT102 (GltT$_{Bs}$; O). Membrane vesicles loaded with 1 mM L-glutamate were diluted 100-fold into 50 mM potassium phosphate, pH 6.0, 5 mM MgSO$_4$ and 3.5 μM L-[^{14}C]-glutamate. The transport reaction was stopped at different time intervals as indicated in the *Experimental procedures*.

accumulation of L-glutamate increased significantly upon the addition of 20 mM NaCl (Fig. 2). Similar observations were made for (sodium)-proton motive force driven L-glutamate uptake in membrane vesicles derived from strain JC5412 harbouring pGBT102 (data not shown). Membrane vesicles derived from strain JC5412 harbouring pGBT102 also showed significantly higher L-glutamate counterflow activity than membrane vesicles derived from strain JC5412 harbouring pUC18 (Fig. 3).

The kinetic parameters (apparent K_m and V_{max}) of L-glutamate uptake in membrane vesicles of *B. caldotenax* and membrane vesicles of *E. coli* JC5412 expressing GltT$_{Bs}$ or GltT$_{Bc}$ were determined (Table 1). Uptake of

Table 1. Apparent kinetic parameters for glutamate transport[a] by membrane vesicles of *B. stearothermophilus*, *B. caldotenax*, *E. coli* JC5412(pGBT102) and JC5412(pGBT231).

Vesicles derived from:		K_m^{app} (μM)	V_{max} (nmol mg protein^{-1} min^{-1})
B. stearothermophilus	(GltT$_{Bs}$)	4.7[b]	11.4[b]
B. caldotenax	(GltT$_{Bc}$)	2.9	17.4
E. coli JC5412(pGBT102)	(GltT$_{Bs}$)	31.8	4.8
E. coli JC5412(pGBT231)	(GltT$_{Bc}$)	25.1	6.2

a. Uptake experiments were performed by diluting membrane vesicles 100-fold in 50 mM potassium phosphate, pH 6.0, 5 mM MgSO$_4$, 40 mM glucose and 500 μM NaCl. After 3 min of preincubation PQQ 920 μM) was added. After another min of incubation L-[^{14}C]-glutamate (1.75 μM) was added and transport assays were further handled as described in the *Experimental procedures*.
b. Data taken from Heyne *et al.* (1991).

glutamate mediated by GltT$_{Bs}$ and GltT$_{Bc}$ yielded lower V_{max} and K_m values when the proteins were assayed in membrane vesicles of *E. coli* relative to membrane vesicles of *B. stearothermophilus* and *B. caldotenax*.

Nucleotide sequence and coding regions of the EcoRI fragments of pGBT102 and pGBT231

The sequencing strategies for the *B. stearothermophilus* and *B. caldotenax* glutamate transport genes are presented in Fig. 4, A and B, respectively. The sequence of the 1537 bp *Eco*RI fragment of *B. stearothermophilus* in pGBT102 is shown in Fig. 5A. Between positions 110 and 1373 an open reading frame (ORF) of 1263 bp is found. The deduced polypeptide contains 421 amino acid residues, corresponding with a molecular mass of 45 469 Da. The nucleotide sequence of the 1535 bp *Eco*RI insert
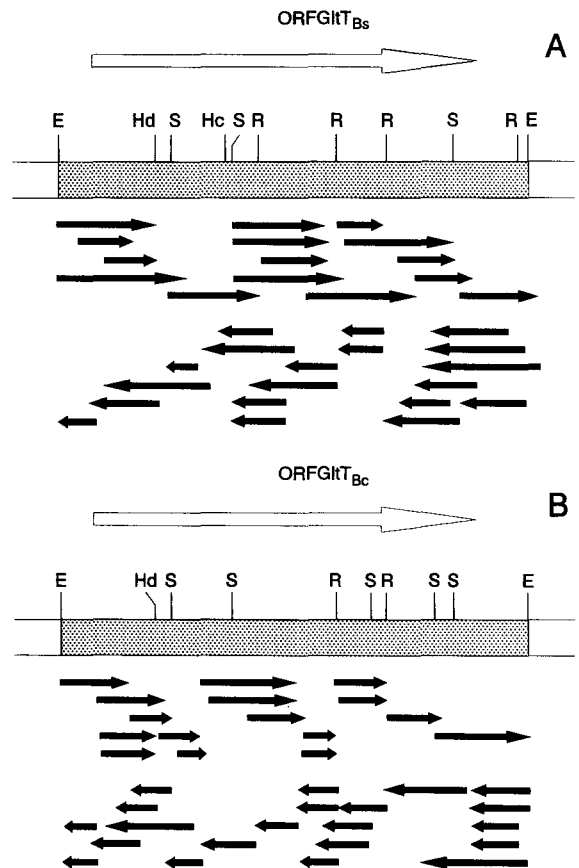
Fig. 4. Sequencing strategy for the *Eco*RI fragments containing the *gltT$_{Bs}$* gene of *B. stearothermophilus* (pGBT102) (A) and the *gltT$_{Bc}$* gene of *B. caldotenax* (pGBT231) (B). Part of the vector sequence (open box), the cloned fragment (shaded box) and the position and direction of transcription of the *gltT$_{Bs/Bc}$* (large arrow above the sequence) are shown. The regions sequenced are indicated by black arrows. Symbols: E, Hd, R, S and Hc represent *Eco*RI, *Hind*III *Rsa*I, *Sau*3A and *Hinc*II restriction endonuclease, respectively.

A

```
                                       -35                         -10
GAATTCAATGACAAATATTATATGAATAATATTCTTGACATAACGAAAATATGATAGTAGAATA              60
                                               START
                                                 M  R  K  I
GTTTTGTCTCTTTTTGTCTAAAAATGTCATAAAGAAAGGGGCGAATGAATGAGAAAAAT               120
                                                 M  R  K  I
TGGATTAGCTTGGCAAATTTTTATTGGTCTCATTCTAGGGATTATCGTGGGAGCCATTTT              180
 G  L  A  W  Q  I  F  I  G  L  I  L  G  I  I  V  G  A  I  F
TTACGGAAATCCGAAAGGTTGCCACTTATTACAGCCTATTGGAGATATTTTCCTTCGTTT              240
 Y  G  N  P  K  V  A  T  Y  L  Q  P  I  G  D  I  F  L  R  L
AATCAAAATGATTGTCATTCCGATTGTTATTTCTAGCCTTGTAGTTGGAGTCGCCAGCGT              300
 I  K  M  I  V  I  P  I  V  I  S  S  L  V  V  G  V  A  S  V
TGGGGATTTGAAGAAGCTTGGAAAATTAGGCGGCAAAACGATTATTTATTTCGAGATTAT              360
 G  D  L  K  K  L  G  K  L  G  G  K  T  I  I  Y  F  E  I  I
CACAACGACGCGGATTGTCGTCGGTTTATTGGCAGCGAATAATTTTCAGCCAGGGACCGG              420
 T  T  I  A  I  V  V  G  L  L  A  A  N  I  F  Q  P  G  T  G
CGTTAATAATGAAATCATTAGAAAAAACCGATATATAAAGCTATGTTGATACAACAACGA              480
 V  N  M  K  S  L  E  K  T  D  I  Q  S  Y  V  D  T  T  N  E
AGTGCAGCATCATTCGATGGTGGAAACTTTGTTAATATTGTTCCAAAAAATATTTTTGA              540
 V  Q  H  S  M  V  E  T  F  V  N  I  V  P  K  N  I  F  E
ATCGTTAACCAAAGGGGATATGCTGCCGATCATTTTCTCTGTTATGTTTCGGTTTAGG               600
 S  L  T  K  G  D  M  L  P  I  I  F  S  V  M  F  G  L  G
AGTAGCGGCGATTGGCGAAAAGAAAGGAAAGCCAGTTCTTCAAGGTACAGCAGA               660
 V  A  A  I  G  E  K  G  K  P  V  L  Q  F  F  Q  G  T  A  E
AGCGATGTTTTATGTAACAAACCAAATTATGAAGTTTTGCGCCGTTTGTTTGCGCT               720
 A  M  F  Y  V  T  N  Q  I  M  K  F  A  P  F  G  V  F  A  L
GATTGGTGTAACCGTTTCTAAGTTTGGGGTAGAGTCGCTTATTCCGCTCAGCAAGCTCGT              780
 I  G  V  T  V  S  K  F  G  V  E  S  L  I  P  L  S  K  L  V
```

```
CATTGTTGTTTACGCAACGATGGTGTTCTTTATCTTTGTCGTGGCCGTGTTGGGCGTGTTGCTAA         840
 I  V  V  Y  A  T  M  V  F  F  I  F  V  V  L  G  G  V  A  K
GTTATTTGGCATAAATATTTTTCATATATATAAAAATTTGAAAGATGAGTTAATTCTTGC            900
 L  F  G  I  N  I  F  H  I  I  K  I  L  K  D  E  L  I  L  A
TTATAGTACAGCAAGTTCGGAAACCGTTCTTCCGAAAATTATGGAGAAAATGGAGAATTT           960
 Y  S  T  A  S  S  E  T  V  L  P  K  I  M  E  K  M  E  N  F
CGGTTGTCCAAAAGCGATTACATCCTTTGTCATTCCGACAGGGTATTCTTTAACTTAGA           1020
 G  C  P  K  A  I  T  S  F  V  I  P  T  G  Y  S  F  N  L  D
CGGTTCTACGTTATATCAGGCGTTGGCGGCCATTTTATCGCGCAGTTGTACGGTTATTGA          1080
 G  S  T  L  Y  Q  A  L  A  A  I  F  I  A  Q  L  Y  G  I  D
CATGCCGATTTCTCAACAAATCTCGCTTTTGCTTGTGTTAATGGTCACGGTTGGGAAT          1140
 M  P  I  S  Q  Q  I  S  L  L  L  V  L  M  V  T  S  K  G  I
CGCTGGGGTGCCGGGTGTATCCTTTGTCGTGCTGCTTGCTACGTTAGGCACGGTTGGGAT         1200
 A  G  V  P  G  V  S  F  V  L  L  A  T  L  G  T  V  G  I
TCCGATAGAAGGATTAGCATTTATCGCTGGAATCGACCGTATTTTAGATATGGCGCAC          1260
 P  I  E  G  L  A  F  I  A  G  I  D  R  I  L  D  M  A  R  T
AGCAGTGAATGTTATTGGCAACTCGTTAGCAGCGATCATTATGTCAAATGGAAGGCCA          1320
 A  V  N  V  I  G  N  S  L  A  A  I  I  M  S  K  W  E  G  Q
ATATAACGAAGAAAAGGAAAAACATCGCGCAATTGCAGCAAAGTGCATAAATGCT           1380
 Y  N  E  E  K  G  K  Q  Y  I  A  Q  L  Q  Q  S  A  -  --
                                                   STOP
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     TERMINATOR
     ----------->
              <-----------
CCACGTAGTAGTATAGTATAATTTGGTGCTGAATAAGGTTGTAATGAGATGCTTGGCTAAGAG         1500
TACATAATACATAGGAGGAAGAACCATCACGAATTC                       1537
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B

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            -35                    -10
                                              START
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                                              M  R  K  I

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G  L  A  W  Q  I  F  I  G  L  I  L  G  I  I  I  V  G  A  I  F

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Y  G  N  P  K  V  A  A  Y  L  Q  P  I  G  D  I  F  L  R  L

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I  K  M  I  V  I  P  I  V  I  S  S  L  V  V  G  V  A  S  V

GGGGATTTGAAAAAGCTTGGAAAAGCGCAAAACGATTATTATTTATTGAGATTATC        300
G  D  L  K  K  K  L  G  G  K  T  I  I  I  Y  F  E  I  I

ACAACGATCGCGATTGTCGTCGGTTTATTGGCGGGCGAATATTTTCAGCCAGGGGCCGGC    360
T  T  I  A  I  V  V  G  L  L  A  A  N  I  F  Q  P  G  A  G

GTTAATATGAAATCATTAGAAAAAACGGATATTCAAAGCTATGTGTGATACAACAAACGAA   420
V  N  M  K  S  L  E  K  T  D  I  Q  S  Y  V  D  T  T  N  E

GTCCAGCATCATTCGATGGTAGAAACATTGTTCAATAATTGTCCGAAAAACATTTTTGAA    480
V  Q  H  H  S  M  V  E  T  F  V  N  I  V  P  K  N  I  F  E

TCGTTATCCACAGGAGACATGCTGCCGATCATTTTCTTCTCTGTTATGTTCCGTTTAGGA   540
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GTAGCGGCGATTGGCGAAAAAGGGAAGCCAGTGCTTCAAGGCACAGCAGAA           600
V  A  A  I  G  E  K  G  K  P  V  L  Q  F  F  Q  G  T  A  E

GCAATGTTTTATGTAACAAACCAAATATGAAGTTTGCCCCGTTCGGTGTGTTTGCGCTT    660
A  M  F  Y  Y  V  T  N  Q  I  M  K  F  A  P  P  G  V  F  A  L

ATCGGTGTAACCGTTTCTAAATTTGGAGTAGAGTCGCTTATTCCGCTCAGCAAGCTCGTC   720
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TTATTTGGTATAAATATTTTTCATATTATAAAAATTTTGAAGATGAATAATTCTTGCT      900
L  F  G  I  N  I  F  H  I  I  K  I  L  K  D  E  L  I  L  A

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Y  S  T  A  S  S  E  T  V  L  P  R  I  M  D  K  M  E  K  F

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G  C  P  K  A  I  T  S  F  V  I  P  T  G  Y  S  F  N  L  D

GGATCTACGTTATATCAGGCGTTGGCTGCCATTTTTATCGCGCAGTTGTACGGTTATTGAC   1080
G  S  T  L  Y  Q  A  L  A  A  I  F  I  A  Q  L  Y  G  I  D

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CCGGTAGAAGGATTAGCATTTATCGCGGGGATCGACCGCATTTTAGATATGGCACGCACA   1260
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                                                            STOP
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TERMINATOR
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------------->          <-------------

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Fig. 5. Nucleotide sequence of the *EcoRI* fragments containing the *gltT*$_{Bs}$ gene of *B. stearothermophilus* (pGBT102) (A) and the *gltT*$_{Bc}$ gene of *B. caldotenax* (pGBT231) (B). The start and stop codons, putative promoter(-35/-10), possible ribosome-binding site (RBS) and possible terminator sequences (→←) are indicated. The amino acid sequence deduced from the DNA sequences of the *gltT*$_{Bs}$ and *gltT*$_{Bc}$ genes are shown below the DNA sequence. The 12 possible membrane-spanning regions are underlined and in bold face. These sequence data appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession numbers M86508 (*gltT*$_{Bs}$) and M86509 (*gltT*$_{Bc}$).

Table 2. Amino acid compositions of the GltT proteins of *B. stearother-mophilus* and *B. caldotenax*.

Amino acid residues	No. or % of amino acids in transport system:			
	GltT$_{Bs}$		GltT$_{Bc}$	
	No.	%	No.	%
Non-polar				
Ala	32	7.6	35	8.3
Cys	1	0.2	1	0.2
Gly	40	9.5	40	9.5
Ile	56	13.3	53	12.6
Leu	42	9.9	44	10.5
Met	15	3.6	15	3.6
Phe	28	6.7	28	6.7
Pro	15	3.6	14	3.3
Trp	2	0.5	2	0.5
Tyr	12	2.9	12	2.9
Val	42	10.0	42	10.0
		Total 67.7		Total 67.9
Polar				
Asp	10	2.4	11	2.6
Glu	16	3.8	16	3.8
Arg	4	1.0	5	1.2
His	3	0.7	3	0.7
Lys	26	6.2	25	5.9
Asn	13	3.1	12	2.9
Gln	17	4.0	16	3.8
Ser	24	5.7	26	6.2
Thr	23	5.5	21	5.0
		Total 32.3		Total 32.1

of plasmid pGBT231 containing the glutamate transport gene of *B. caldotenax* is shown in Fig. 5B. An ORF is found between positions 109 and 1372, encoding a polypeptide of 421 amino acid residues (molecular mass 45 345 Da).

Amino acid composition, sequence homology and hydropathy

The amino acid compositions of GltT$_{Bs}$ and GltT$_{Bc}$ are shown in Table 2. GltT$_{Bs}$ contains 67.7% non-polar and 32.3% polar residues, indicating a composition typical of membrane proteins (Büchel *et al.*, 1980). Of the 421 residues present in GltT$_{Bs}$, 30 (7.2%) are basic (His residues were not taken into account) and 26 (6.2%) were acidic. The GltT$_{Bs}$ protein is therefore a basic protein with an excess of four positive charges at neutral pH. The theoretical isoelectric point is 9.3. Similar data were obtained for the GltT$_{Bc}$ protein, although this protein has an excess of three positive charges at neutral pH and a theoretical isoelectric point of 9.1. Alignment of the nucleotide sequences of *gltT*$_{Bs}$ and *gltT*$_{Bc}$ revealed 72 mismatches (in 68 triplets). However, they result in only 14 mismatches at amino acid level, i.e. 96.7% identical amino acid residues (Fig. 6 and Table 3). The deduced amino acid sequences of the Na$^+$/H$^+$/glutamate symport proteins of *B. stearothermophilus* and *B. caldotenax* were compared with the revised sequence of the H$^+$/glutamate

symport protein of *E. coli* K-12 (Tolner *et al.*, 1992) and the sequence of the Na$^+$/glutamate symport proteins of *E. coli* B (Deguchi *et al.*, 1990) and *E. coli* K-12 (Kalman *et al.*, 1991). Sequence comparisons revealed extensive similarity between the thermophilic Na$^+$/H$^+$/glutamate symport proteins and the H$^+$/glutamate symport system of *E. coli*, comprising 57.2% identity (Fig. 6 and Table 3). There was no significant similarity between the thermophilic Na$^+$/H$^+$/glutamate symport proteins and the Na$^+$/glutamate symport proteins of *E. coli* B and K-12 (Table 3). Also, no similarity was found between the glutamate transport proteins of the thermophilic bacilli and any other protein in the SWISSPROT Protein Sequence Data Bank (Version 1.40), except for some local similarity with other Na$^+$-dependent transport proteins.

The method of Eisenberg *et al.* (1984) predicts, for both thermophilic proteins, 12 membrane-spanning regions (Fig. 7). The 12 membrane-spanning regions of GltT$_{Bs}$ and GltT$_{Bc}$ are located in similar positions as the 12 membrane-spanning segments predicted for the *E. coli* H$^+$/glutamate transport protein (Fig. 7), although the putative membrane-spanning helices 4 and 12 in GltT$_{Bs}$ and GltT$_{Bc}$ do have a somewhat lower hydrophobicity than the corresponding regions in GltP$_{Ec}$.

Codon usage

The codon usage in the *gltT*$_{Bs}$ and *gltT*$_{Bc}$ genes is nearly identical (Table 4). The low-GC content of the *gltT*$_{Bs}$ and *gltT*$_{Bc}$ genes (40.3 and 40.7%, respectively), when compared with the *gltP*$_{Eck12}$ gene (53.2%), is reflected in the codon usage. At all codon positions, but particularly at the third position, a strong preference for A or U over G or C can be seen.

Discussion

Uptake of L-glutamate and L-aspartate by whole cells and membrane vesicles of *E. coli* JC5412 harbouring pGBT102 (GltT$_{Bs}$) increased significantly upon addition of 20 mM sodium (Fig. 2). These results are in accordance with those of glutamate transport in membrane vesicles of

Table 3. Amino acid identity (similarity) between pairs of proteins.

Protein	% Amino Acid Identity (Similarity) With:			
	GltT$_{Bs}$	GltT$_{Bc}$	GltP$_{Eck-12}$	GltS$_{Eck-12/EcB}$
GltT$_{Bs}$	100			
GltT$_{Bc}$	96.7	100		
	(2.1)			
GltP$_{EcK-12}$	60.1	60.3	100	
	(17.3)	(16.9)		
GltS$_{EcK-12/EcB}$	8.5	9.5	12.5	100
	(6.0)	(6.5)	(8.5)	

```
                                                    #       S
GltT_Bs    MRKI--GLAWQIFIGLILGIIVGAI--FYGNPK---VATYLQPIGDIFLR    43
GltT_Bc    MRKI--GLAWQIFIGLILGIIVGAI--FYGNPK---VAAYLQPIGDIFLR    43
GltP_Ec    MKNIKFSLAWQILFAMVLGILLGSYLHYHSDSRDWLVVNLLSPAGDIFIH    50
           *..*  .*****.....***..*.     ......    *.. * * ****..

                         S   S   SS
GltT_Bs    LIKMIVIPIVISSLVVGVASVGDLKKLGKLGGKTIIYFEIITTIAIVVGL    93
GltT_Bc    LIKMIVIPIVISSLVVGVASVGDLKKLGKLGGKTIIYFEIITTIAIVVGL    93
GltP_Ec    LIKMIVVPIVISTLVVGIAGVGDAKQLGRIGAKTIIYFEVITTVAIILGI   100
           ******.*****.****.*.*** *.**..*.*.*******.***.**..*.

                  #
GltT_Bs    LAANIFQPGTGVNMKSLEKTDIQSYVDTTNEVQH--HSMVETFVNIVPKN   141
GltT_Bc    LAANIFQPGAGVNMKSLEKTDIQSYVDTTNEVQH--HSMVETFVNIVPKN   141
GltP_Ec    TLANVFQPGAGVDMSQLATVDISKYQSTTEAVQSSSHGIMGTILSLVPTN   150
           **.****.**.*. *...** .* .**..**    *....*....**.*

                     ##
GltT_Bs    IFESLTKGDMLPIIFFSVMFGLGVAAIGEKGK-PVLQFFQGTAEAMFYVT   190
GltT_Bc    IFESLSTGDMLPIIFFSVMFGLGVAAIGEKGK-PVLQFFQGTAEAMFYVT   190
GltP_Ec    IVASMAKGEMLPIIFFSVLFGLGLSSLPATHREPLVTVFRSISETMFKVT   200
           * .*...*.*********.****.... .. . *.. *....*.** **

                                      #       #
GltT_Bs    NQIMKFAPFGVFALIGVTVSKFGVESLIPLSKLVIVVYATMVFFIFVVLG   240
GltT_Bc    NQIMKFAPFGVFALIGVTVSKFGVESLIPLSKLVIVVYATMLFFIFAVLG   240
GltP_Ec    HMVMRYAPVGVFALIAVTVANFGFSSLWPLAKLVLLVHFAILFFALVVLG   250
           . .*..** ******.***..** .** **.***...*. ...** ..***

                                   #   #   #
GltT_Bs    GVAKLFGINIFHIIKILKDELILAYSTASSETVLPKIMEKMENFGCPKAI   290
GltT_Bc    GVAKLFGINIFHIIKILKDELILAYSTASSETVLPRIMDKMEKFGCPKAI   290
GltP_Ec    IVARLCGLSVWILIRILKDELILAYSTASSESVLPRIIEKMEAYGAPVSI   300
           **.* *.....*.***************.***.*..*** .* * .*

                                      ##
GltT_Bs    TSFVIPTGYSFNLDGSTLYQALAAIFIAQLYGIDMPISQQISLLLVLMVT   340
GltT_Bc    TSFVIPTGYSFNLDGSTLYQALAAIFIAQLYGIDMSVSQQISLLLVLMVT   340
GltP_Ec    TSFVVPTGYSFNLDGSTLYQSIAAIFIAQLYGIDLSIWQEIILVLTLMVT   350
           ****.***************..************... *.* *.*.****

                               #
GltT_Bs    SKGIAGVPGVSFVVLLATLGTVGIPIEGLAFIAGIDRILDMARTAVNVIG   390
GltT_Bc    SKGIAGVPGVSFVVLLATLGTVGIPVEGLAFIAGIDRILDMARTAVNVIG   390
GltP_Ec    SKGIAGVPGVSFVVLLATLGSVGIPLEGLAFIAGVDRILDMARTALNVVG   400
           *******************.****.*******.**********.**.*

                               # #
GltT_Bs    NSLAAIIMSKWEGQYNEEKGKQY----IAQLQQSA--    421
GltT_Bc    NSLAAIIMSKWEGQYNEEKGKQY----LAELQQSA--    421
GltP_Ec    NALAVLVIAKWEHKFDRKKALAYEREVLGKFDKTADQ   437
           *.**.....*** ... .*. .*    .......*
```

Fig. 6. Multiple alignment of the deduced amino acid sequences of the GltT_Bs, GltT_Bc and GltP_EcK-12 glutamate transport proteins. The best fit was achieved by introducing gaps in order to maximize the identity score. The overall identity was 57.2%. Identical residues and conserved substitutions are indicated by asterisks and full points, respectively. Symbols: S, residues involved in the putative Na+ recognition or binding motif; #, mismatches between the GltT_Bs and GltT_Bc proteins. GltP_EcK-12 sequence was taken from Tolner *et al.* (1992).

B. stearothermophilus (de Vrij *et al.*, 1989; Heyne *et al.*, 1991) and therefore suggest that the gene encoding the previously described sodium/proton/glutamate symport protein of *B. stearothermophilus* (de Vrij *et al.*, 1989; Heyne *et al.*, 1991) has been cloned.

The GltT_Bs and GltT_Bc proteins both consist of 421 amino acid residues, corresponding with molecular masses of 45 469 and 45 345 Da, respectively. These values are higher than the apparent molecular masses of 33 000 Da estimated from SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1). However, aberrant electrophoretic behaviour is often observed for integral membrane proteins (e.g. Büchel *et al.*, 1980; Deguchi *et al.*, 1990; Ehring *et al.*, 1980; Nakao *et al.*, 1987; Poolman *et al.*, 1989; Van der Rest *et al.*, 1990; Wallace *et al.*, 1990; Yazyu *et al.*, 1984), and is probably explained by increased binding of sodium dodecyl sulphate due to the hydrophobic nature of the proteins.
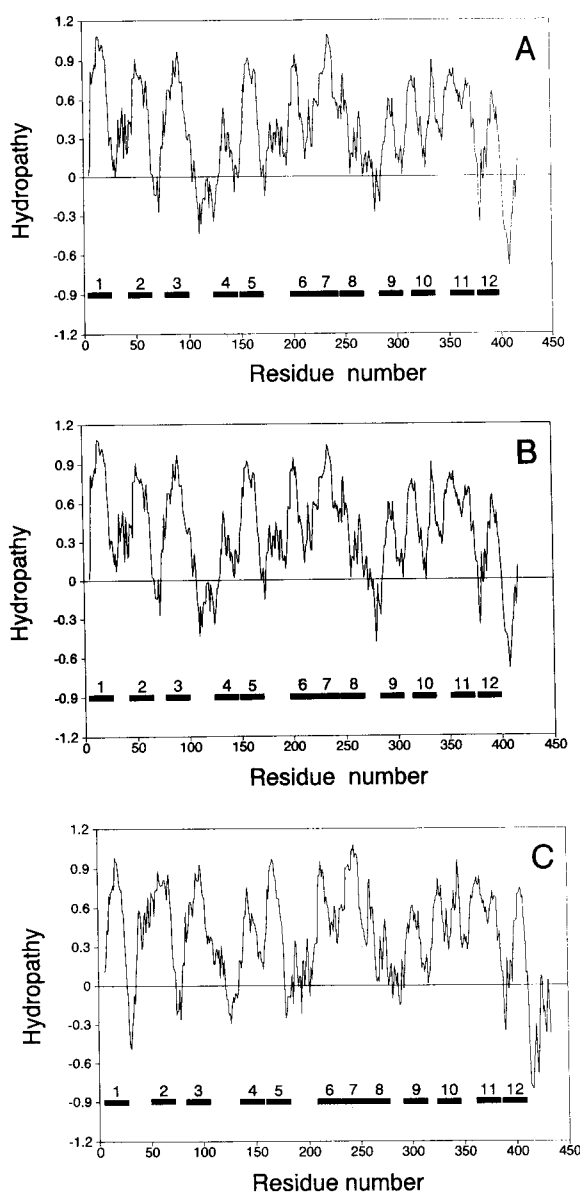
Fig. 7. Hydropathy profiles of the GltT$_{Bs}$ (A), GltT$_{Bc}$ (B) and GltP$_{EcK-12}$ (C) glutamate transport proteins. The hydropathy was calculated according to the method of Eisenberg *et al.* (1984) with a window of 21 amino acids. The positions of the 12 putative membrane-spanning segments are indicated by solid bars. GltP$_{EcK-12}$ sequence was taken from Tolner *et al.* (1992).

Transcription of *gltT*$_{Bs}$ is probably initiated from the promoter indicated in Fig. 5A. The −35 and −10 promoter regions, and the spacing of 17 bp between the two regions, is similar to that of consensus promoter sequences (−35: TTGACA; −10: TATAAT; spacing 16–18, 17–19 bp) that are recognized by the σ^{43} and σ^{70} factors of the holoenzyme form of RNA polymerase of *B. subtilis* (Helmann and Chamberlin, 1988; Moran *et al.*, 1982) and *E. coli* (Hawley and McClure, 1983; Helmann and Chamberlin, 1988), respectively. This suggests the

existence of a sigma factor in *B. stearothermophilus*, which is similar to σ^{43} in *B. subtilis* and σ^{70} in *E. coli*, that are involved in transcription of genes for housekeeping functions. A putative ribosome-binding site (RBS) is located at proper distance (4 bp) from the translation initiation codon (Fig. 5A). The RBS shows extensive complementarity to the 3' end of *B. stearothermophilus* 16S rRNA (Douthwaite *et al.*, 1983). The stop codon (TAA at position 1373–1375) is followed by an inverted repeat (ΔG° −114.2 kJ mol^{-1}, calculated according to Tinoco *et al.*, 1973) with features of a putative rho-independent transcription terminator sequence (Rosenberg and Court, 1979). The −35 and −10 promoter regions of *gltT*$_{Bc}$ are identical to those of *gltT*$_{Bs}$. The putative ribosomal binding site (Fig. 5B), however, shows major differences and is probably much weaker than the one upstream of *gltT*$_{Bs}$ of *B. stearothermophilus*. Also, the transcription terminator sequence is much weaker when compared with the one in the *B. stearothermophilus* glutamate transport gene (ΔG° −75.6 kJ mol^{-1}, calculated according to Tinoco *et al.*, 1973). The differences in the expression signals of *gltT*$_{Bs}$

Table 4. Codon usage of the glutamate transport gene of *B. stearothermophilus* (*Bs*) and *B. caldotenax* (*Bc*).

Codon	Amino Acid	No. of times used Bs	(Bc)	Codon	Amino Acid	No. of times used Bs	(Bc)
TTT	Phe	19	(20)	TAT	Tyr	8	(10)
TTC	Phe	9	(8)	TAC	Tyr	4	(2)
TTA	Leu	17	(17)	TAA	---	1	(1)
TTG	Leu	7	(8)	TAG	---	0	(0)
CTT	Leu	11	(12)	CAT	His	3	(3)
CTC	Leu	3	(4)	CAC	His	0	(0)
CTA	Leu	1	(0)	CAA	Gln	11	(10)
CTG	Leu	3	(3)	CAG	Gln	6	(6)
ATT	Ile	40	(36)	AAT	Asn	8	(6)
ATC	Ile	13	(14)	AAC	Asn	5	(6)
ATA	Ile	3	(3)	AAA	Lys	18	(18)
ATG	MET	15	(15)	AAG	Lys	8	(7)
GTT	Val	17	(16)	GAT	Asp	7	(7)
GTC	Val	8	(9)	GAC	Asp	3	(4)
GTA	Val	6	(7)	GAA	Glu	11	(13)
GTG	Val	11	(10)	GAG	Glu	5	(3)
TCT	Ser	6	(5)	TGT	Cys	1	(0)
TCC	Ser	2	(5)	TGC	Cys	0	(1)
TCA	Ser	2	(3)	TGA	---	0	(0)
TCG	Ser	7	(6)	TGG	Trp	2	(2)
CCT	Pro	1	(0)	CGT	Arg	2	(1)
CCC	Pro	0	(0)	CGC	Arg	1	(2)
CCA	Pro	4	(5)	CGA	Arg	0	(0)
CCG	Pro	10	(9)	CGG	Arg	0	(0)
ACT	Thr	3	(1)	AGT	Ser	3	(3)
ACC	Thr	5	(2)	AGC	Ser	4	(4)
ACA	Thr	9	(12)	AGA	Arg	1	(2)
ACG	Thr	6	(6)	AGG	Arg	0	(0)
GCT	Ala	6	(7)	GGT	Gly	10	(10)
GCC	Ala	4	(5)	GGC	Gly	10	(10)
GCA	Ala	8	(11)	GGA	Gly	12	(11)
GCG	Ala	14	(12)	GGG	Gly	8	(9)

and *gltT*$_{Bc}$ are not reflected in the expression levels of the proteins both in *E. coli* and the thermophilic bacilli (Table 1). Since the expression levels of GltT in *B. stearothermophilus* and *B. caldotenax* are similar, the observed differences in growth rates on glutamate as sole carbon, energy and nitrogen source cannot be explained at the level of transport. The presence of putative promoter and transcription termination sequences flanking the glutamate transport genes of *B. stearothermophilus* and *B. caldotenax* suggests that both genes are transcribed as single cistronic messages.

The apparent K_m and V_{max} values for L-glutamate transport in membrane vesicles derived from *B. stearothermophilus* and *B. caldotenax* are very similar (Table 1). However, *B. caldotenax* can grow approximately 10-fold faster than *B. stearothermophilus* in media with 50 mM glutamate as sole source of energy, carbon and nitrogen (data not shown). Therefore it is unlikely that the GltT$_{Bs}$ transport protein is limiting the growth of *B. stearothermophilus* on glutamate as sole source of energy, carbon and nitrogen.

The deduced amino acid sequence of the Na$^+$/H$^+$/glutamate symport proteins of *B. stearothermophilus* and *B. caldotenax* were initially compared with the H$^+$/glutamate symport protein of *E. coli* K-12 (Wallace *et al.*, 1990). This did reveal regions of homology while other regions differed completely. By translating the nucleotide sequence of *gltP*$_{EcK-12}$ in different reading frames, and by comparing the translated sequences with those of GltT$_{Bs}$ and GltT$_{Bc}$ it became apparent that the sequence divergence between GltP$_{EcK-12}$ and GltT$_{Bs}$ and GltT$_{Bc}$ was probably caused by sequencing errors, i.e. base substitutions, deletions and insertions, in the L-glutamate transport gene of *E. coli*. The *gltP*$_{EcK-12}$ sequence, as published by Wallace *et al.* (1990), was therefore resequenced and revised (Tolner *et al.*, 1992). Sequence comparisons revealed extensive similarity between the GltT$_{Bs}$ and GltT$_{Bc}$ and the revised GltP$_{EcK-12}$ sequence (Fig. 6 and Table 3).

A conserved amino acid sequence has been proposed to be involved in Na$^+$ recognition or binding (SOB-motif __-G-__-A----L---GR-__) (Deguchi *et al.*, 1990). This sequence was also found in the GltT$_{Bs}$ and GltT$_{Bc}$ proteins, except that Arg was replaced for Lys (__-G$_{38}$-__-A$_{62}$----L$_{67}$---G$_{71}$K$_{72}$__; see also Fig. 6). However, apart from one mismatch, the SOB motif can also be found in the GltP$_{Ec}$ protein (__-G$_{45}$-__-A$_{69}$----A$_{74}$----G$_{78}$R$_{79}$__; see also Fig. 6). If indeed this SOB motif is essential for Na$^+$-binding this mismatch could explain the inability of GltP$_{Ec}$ to use Na$^+$ as coupling ion despite the extensive similarity between GltT$_{Bs}$ and GltP$_{Ec}$. On the other hand, the SOB motif of GltT$_{Bs}$ and GltT$_{Bc}$ is located in a short hydrophilic region and might be involved in retention of the three-dimensional shape of these proteins, as is proposed for

this region in the 'consensus glucose transport protein' (Henderson, 1990).

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains, plasmids and phages used in this study are listed in Table 5. *B. stearothermophilus* and *B. caldotenax* were grown at 63 and 70°C, respectively, with vigorous aeration in a medium containing 2% (w/v) tryptone, 1% (w/v) yeast extract and 170 mM NaCl, and adjusted to pH 7.0. For growth experiments, mineral medium of pH 7.0 was used, containing 1 ml of trace element solution (Vishniac and Santer, 1957) per litre of medium, 34 mM Na$_2$HPO$_4$, 22 mM KH$_2$PO$_4$, 10 mM NaCl, 1 mM MgSO$_4$, 0.1 mM CaCl$_2$, and 50 mM L-glutamate as sole source of energy, nitrogen and carbon. *E. coli* strains were grown at 37°C with vigorous aeration in LB, M9, M9G (M9 in which ammonium-chloride was replaced by L-glutamate at a final concentration of 10 mM) or M9CA medium (Sambrook *et al.*, 1989). The mineral media were supplemented with essential nutrients as indicated by the auxotrophic markers. When needed, carbenicillin and IPTG were added to a final concentration of 100 µg ml^{-1} and 100 µM, respectively.

DNA manipulations

Mini- and large-scale preparations of plasmid DNA were obtained by the alkaline lysis method (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981). Chromosomal DNA was isolated essentially as decribed previously (Leenhouts *et al.*, 1990), except that mutanolysine was omitted. The strains were transformed by the rubidium chloride (Sambrook *et al.*, 1989) or by the electrotransformation (Dower *et al.*, 1988) method. Other DNA techniques were performed as decribed previously (Sambrook *et al.*, 1989).

Cloning of the glutamate transport gene

The strategy for cloning the *gltT*$_{Bs}$ and *gltT*$_{Bc}$ genes is based on the complementation of *E. coli* K-12 strain JC5412, which does not grow on glutamate as sole source of energy, nitrogen and carbon. Partially *Eco*RI-, *Hind*III-, *Pst*I- or *Sau*3A-digested chromosomal DNA of *B. stearothermophilus* or *B. caldotenax* was fractionated by polyacrylamide gel (6% w/v) electrophoresis. Fragments of 2 to 20 kb were electroeluted from the gel and ligated into linearized and dephosphorylated pKK223–3. The resulting hybrid plasmids were used to transform *E. coli* JC5412 by electrotransformation. Transformants able to grow on M9G plates (supplemented with carbenicillin and IPTG) were analysed with respect to their plasmid content. Purified plasmids were used to retransform *E. coli* JC5412 in order to distinguish between Glu$^+$ revertants and true transformants. Transformants again were selected on M9G plates.

Sequence determination of the glutamate transport genes

The nucleotide sequences of both strands of the *Eco*RI fragment of pGBT102 and pGBT231, or subclones derived thereof

Table 5. Bacterial strains, plasmids and phages used.

Bacterial stain, plasmid or phage	Relevant characteristics	Source/Reference
Bacterium		
B. stearothermophilus		ATCC7954
B. caldotenax		Heinen and Heinen (1972)
E. coli		
JM101	Δ(lac–proAB) (F' lacI^q ΔM15)	Yanisch-Perron et al. (1985)
JC5412	doesn't grow on L-glutamate as sole carbon, nitrogen and energy source	Willetts and Clark (1969)
P678-54	Minicell-producing	Adler et al. (1967)
Plasmid		
pUC18	Ap^R	Yanisch-Perron et al. (1985)
pKK223-3	Ap^R, expression vector	Pharmacia
pGBT38	pKK223-3, carrying gltT of B. stearothermophilus on a 2500 bp EcoRI–EcoRI fragment	This work
pGBT102	pUC18, carrying gltT of B. stearothermophilus on a 1537 bp EcoRI–EcoRI fragment	This work
pGBT112	pUC18, carrying gltT of B. stearothermophilus on a 1537 bp EcoRI–EcoRI fragment (in reverse orientation relative to pGBT102)	This work
pGBT231	pUC18, carrying gltT of B. caldotenax on a 1535 bp EcoRI–EcoRI fragment	This work
Phage		
M13mp18/19		Yanisch-Perron et al. (1985)

Ap^R, ampicillin-resistant.

in pUC18 or M13mp18/19 (RsaI, Sau3A, HpaI, HindIII or HincII fragments), were determined by using the dideoxy-chain termination method (Sanger et al., 1977). A T7 sequencing kit (Pharmacia) was used in sequencing either single- or double-stranded DNA. MICROGENIE (Release 5.0, Beckman, Palo Alto, Cal., USA) and PCGENE (release 6.26, Genofit) were used for computer-assisted sequence analysis.

Transport assays with whole cells

Cells (15 ml) of strain JC5412 harbouring plasmid pUC18, pGBT102, or pGBT231, grown for 10 h in LB (supplemented with carbenicillin and IPTG), were harvested, washed three times in 50 mM potassium phosphate, pH 6.0, and 5 mM MgSO$_4$ and resuspended to a final A$_{660}$ of approximately 300 in the same buffer. Uptake of L-[^{14}C]-glutamate or L-[^{14}C]-aspartate was assayed at 37°C, upon 100-fold dilution of the cells into buffer consisting of 50 mM potassium phosphate, pH 6.0, 5 mM MgSO$_4$, and 10 mM glucose, with or without the addition of 20 mM NaCl or 20 mM KCl (see legends for details). This mixture was incubated for 1 min at 37°C under continuous aeration. To initiate the uptake experiment L-[^{14}C]-glutamate or L-[^{14}C]-aspartate was added to a final concentration of 1.75 or 2.23 μM, respectively. The uptake reactions were terminated by adding a 20-fold excess of ice-cold 0.1 M potassium chloride, followed by immediate filtration over cellulose nitrate filters (0.45 μm, pore size). The filters were washed once with 2 ml of ice-cold potassium chloride.

Transport assays with membrane vesicles

For transport studies in membrane vesicles, cells of strain JC5412 harbouring plasmid pUC18, pGBT102, or pGBT231, were grown to an A$_{660}$ of 0.7 in LB (supplemented with carbenicillin and IPTG). Cells were harvested and membrane vesicles were isolated as described previously (Kaback, 1971). Membranes were finally resuspended to 15 mg protein per ml in 50 mM potassium phosphate, pH 6.0, and stored in liquid nitrogen.

Modes of transport

Counterflow activity. Membrane vesicles were washed twice with 50 mM potassium phosphate, pH 6.0, 5 mM MgSO$_4$ and resuspended in the same buffer supplemented with 1 mM L-glutamate. After 2 h of incubation at room temperature, membrane vesicles were pelleted by centrifugation and resuspended to 20 mg protein per ml in the same buffer. Counterflow was initiated by diluting membrane vesicles 100-fold with buffer consisting of 50 mM potassium phosphate, pH 6.0, 5 mM MgSO$_4$ and 3.5 μM L-[^{14}C]-glutamate. The reaction was terminated as described for whole cells.

Sodium/proton motive force driven uptake. The electron donor system 2,7,9, tricarboxy-1-H-pyrrolo-(2,3)-quinoline-4,5-dione (PQQ)/glucose was used to generate a Δp (van Schie et al., 1985). Membrane vesicles were diluted 100-fold with buffer consisting of 50 mM potassium phosphate, pH 6.0, 5 mM

MgSO$_4$, and 40 mM glucose. After 3 min of preincubation the electron mediator PQQ was added to a final concentration of 20 μM. To initiate the uptake experiment, L-[^{14}C]-glutamate or L-[^{14}C]-aspartate was added after another minute of incubation to a final concentration of 1.75 and 2.23 μM, respectively. Further handling was the same as described for whole cells. All transport assays were carried out at 37°C.

Determination of kinetic parameters

The kinetic parameters for transport, apparent K_m and V_{max}, were estimated from the uptake of labelled amino acid in the first 10 s. Results were analysed by Eadie–Hofstee Plots.

Minicells

Minicells of strain P678–54 were purified in three subsequent sucrose gradient centrifugations (Maeger *et al.*, 1977). The *in vivo* labelled ([^{35}S]-methionine) proteins were resolved by 15% (w/v) (SDS–PAGE) and visualized by autoradiography.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Nomenclature

In order to discriminate between the proton/glutamate and the sodium/glutamate transport proteins of *E. coli* the gene designations *gltP* and *gltS* are used. The L-glutamate transport systems of *B. stearothermophilus* and *B. caldotenax* translocate glutamate in symport with sodium ions and protons. For the gene encoding these proteins the designation *gltT* was used. To discriminate between the genes and proteins the subscripts Bs, Bc and Ec (B or K-12) were added, for *B. stearothermophilus*, *B. caldotenax* and *E. coli* (B or K-12), respectively.

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